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Identification of mRNAs encoding two different soluble forms of the human interferon α-receptor

Carolina Abramovich[a], Edward Ratovitski[a], Erik Lundgren[b], Michel Revel[a,*]

[a]*Department of Molecular Genetics and Virology, Weizmann Institute of Science, Rehovot 76100, Israel*
[b]*Institute of Cellular Molecular Biology, Umea Universitet, Umea S-901 87, Sweden*

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Abstract

Transcripts of the human IFN α-receptor (IFNAR) gene, lacking the transmembrane (TM) domain were found in human myeloma U266^S cells, in addition to the transmembranal IFNAR cDNA Two different cDNAs encoding such soluble IFNAR forms were identified Form 1 has a deletion causing a frameshift toward the end of the extracellular (EC) domain predicting a tail of 7 amino acids Form 2 has two in-frame deletions and conserves most of the intracytoplasmatic domain of IFNAR The transcripts for the two soluble forms are still found in U266^R cells which have lost the transmembranal IFNAR transcript Human cells seem to have independent mechanisms to synthesize soluble IFN receptors, which may act as competitors outside the cells or carry IFN-mediated functions inside the cell

Key words Interferon receptor, Soluble receptor, Alternative splicing, Myeloma cell

1. Introduction

Cytokines, among which interferons (IFNs), are cell secreted proteins which typically act from the outside of cells through specific plasma membrane receptors As a common feature, these receptors have a ligand-binding extracellular (EC) domain, separated from an intracytoplasmatic (IC) domain by an internal sequence of hydrophobic amino acids (aa), the transmembrane (TM) domain, which anchors the receptor in the cell outer membrane [1] In addition, forms of cytokine receptors lacking the TM and IC domains (soluble receptors) have been characterized (see [2] for review) Such forms may result from proteolytic cleavage between the EC and TM domains [3,4]. However, soluble receptors of several cytokines were shown to be encoded by mRNAs arising from differential processing of the receptor gene transcripts, indicating that these forms are synthesized by the cells along with the transmembranal receptors [5,6]

Cloning of the IFN α-Receptor (IFNAR) cDNA [7] revealed a transmembranal protein with a 21 amino acid-long hydrophobic TM region, an N-terminal EC domain (436 aa) involved in ligand binding [8,9] and a C-terminal IC domain (100 aa) Recently, Novick et al. [8] reported the presence of soluble IFNAR molecules in body fluids. To investigate the existence of gene transcripts encoding soluble IFNAR forms, we analyzed RNA from human myeloma U266 cells, by reverse transcription and polymerase chain reaction (PCR) Besides the transmembranal IFNAR cDNA, we identified two additional mRNAs which encode different soluble IFNAR forms, one truncated after the EC domain, the other with in-phase deletions conserving both EC and IC domains

2. Materials and methods

2 1 *Cell cultures*

Human myeloma cells U266 (American Type Culture Collection, Rockville, MD), or U266^S, were cultured in RPMI 1640 (Biolabs, Israel), supplemented with 10% heat-inactivated fetal calf serum, at 37°C in 5% CO_2 The U266^R cells have been derived by long term culture in increasing concentrations of human IFN-α, up to 2,000 IU/ml, and these IFN-resistant cells grow with the same doubling time as the original U266^S cells

2 2 *RNA extraction, cDNA preparation, primers and PCR*

Total cell RNA was prepared by the guanidine thiocyanate method [10], and cDNA was synthesized with reverse transcriptase (Boehringer, Mannheim) using oligonucleotide primer 5, complementary to residues 1,729–1,749 of the IFNAR cDNA sequence [7], and according to published protocols [11] The cDNA reactions were diluted 1 5 in water, appropriate oligonucleotide primers were added and PCR amplification was performed with Taq polymerase (Promega Biotec, Madison, WI), with 35 cycles of 1 min at 92°C, 1 min at 55°C and 1 min at 72°C

The following primers were used (added restriction sites in lower case and coordinates numbered as in IFNAR cDNA, ref [7]) Primer 1 (sense 1,270–1,290) 5′-gctcgaattCCACTGACTGTATATTGTGTG, Primer 2 (sense 1,342–1,361) 5′-AGTGACGCTGTATGTGAGAA, Primer 2a (sense 1,331–1,343) 5′-acgtgaattcGCAGTGTTTTTAG, Primer 2b (sense 1,320–1,336) 5′-gatcgaattcGCTGAATAAAAG-CAGTG, Primer 3 (antisense 1,498–1,515) 5′-ATCTATACTG-GAAGAAGG, Primer 4 (sense 1,522–1,542) 5′-TTCTCTGAACAGC-CATTGAAG, Primer 5 (antisense 1,729–1,749) 5′-TACAAAG-TCCTGCTGTAGTTC, Primer 5a (antisense 1,733–1,752) 5′-cgaag-

*Corresponding author Fax (972) (8) 344108

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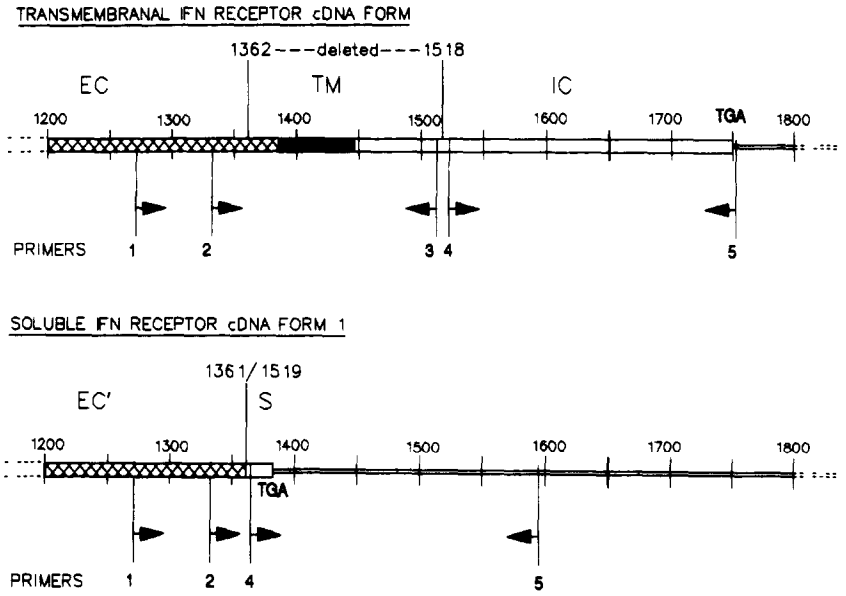

Fig 1 (upper) Scheme of part of the transmembranal IFNAR cDNA [7] with end of EC domain (cross-hatched box), TM domain (black box) and IC domain (open box) Position of some of the PCR primers shown by arrows (right pointed, sense, left-pointed, antisense) The deletion in soluble IFNAR cDNA form 1 is indicated as deduced from sequence in Fig 3 (lower) Scheme of same region in form 1 Novel S domain of seven C-terminal amino acids shown by open box

gatccTCATACAAAGTCCTGCTGTA, Primer 6 (antisense 1,601–1,619) 5'-ACTGTAGCAATTGTGCTTA

2 3 Cloning and sequencing of PCR products

For cloning, PCR products were synthesized with primers starting with tails containing *Eco*RI sites (sense primers 1, 2a, 2b) and *Bam*HI sites (antisense primer 5a) The products were digested with *Eco*RI and *Bam*HI, separated on 2% agarose gels, purified and cloned in the BlueScript KS$^+$ vector (Stratagene Cloning Systems, LaJolla, CA) which had been cut by the same restriction enzymes Plasmids with PCR DNA inserts were sequenced using the Sequenase Version 2 0 DNA sequencing kit (USB, Cleveland, OH) as described [12] Sequencing was done from the T3 primer of the BlueScript DNA and the complementary strand from primer 6 (antisense 1,601–1,619)

3. Results

3 1 Multiple IFNAR cDNAs from U266 cell RNA

From RNA of human myeloma U266^S cells, single stranded cDNA was synthesized by priming with an oligonucleotide (primer 5 in Fig 1) complementary to the end of the IFNAR cDNA coding region (residues 1,729–1,749) reported by Uze et al [7] The cDNA was subjected to PCR amplification with three pairs of sense and antisense primers, positioned on the sequence of the transmembranal IFNAR cDNA, as illustrated in the upper part of Fig. 1 The PCR DNA products were analyzed by agarose gel electrophoresis and ethidium bromide staining (Fig 2) With primers 2 and 5 (spanning residues 1,342 to 1,749), two bands of amplified DNA were observed (Fig 2, lane 3) the longer one of about 420 bp, was in the range expected for the transmembranal IFNAR cDNA, the second product being shorter and about 260 bp That this shorter DNA fragment originates from a different transcript, was indicated by the study of RNA from U266^R cells, a variant isolated by resistance to growth inhibition by IFN-α With primers 2 and 5, U266^R cDNA gave only the 260 bp PCR product, in the absence of the 420 bp product (Fig 2, lane 4) This result suggested that the 260 bp product corresponds to a separate deleted transcript produced by both S and R U266 cell types, the latter cells lacking the full length IFNAR transcript. Several other pairs of

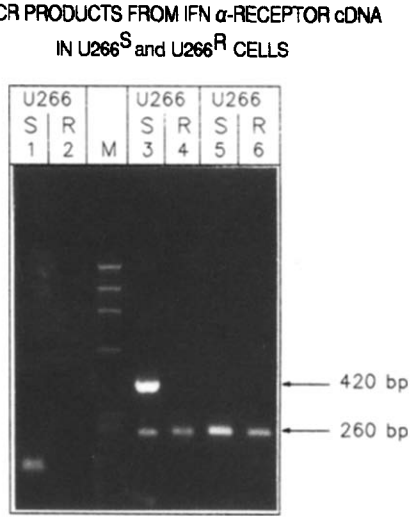

Fig 2 Polymerase chain reaction (PCR) products of cDNA from U266^S (lanes 1,3,5) and U266^R (lanes 2,4,6) cellular RNA, with primers indicated in Fig 1 Lanes 1,2 primers 2 and 3 Lanes 3,4 primers 2 and 5 Lanes 5,6 primers 4 and 5 M, ϕX174 DNA *Hae*III digest markers Ethidium bromide stained agarose gel electrophoresis Approximated size of products shown by arrows

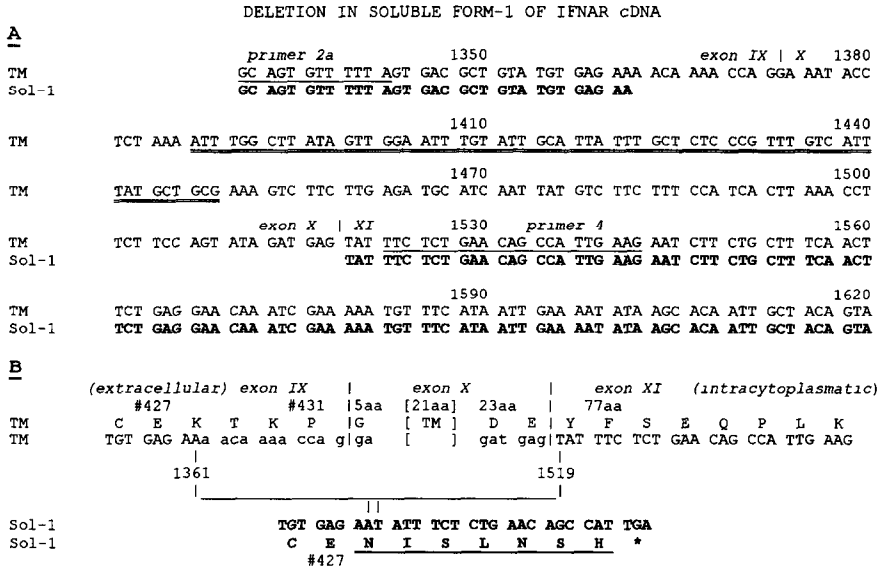

Fig 3 (A) Partial nucleotide sequence of cloned PCR products made with primers 2a and 5 TM designates the sequence of the larger product of about 420 bp (similar to Fig 2, lane 3) identical to the transmembranal IFNAR cDNA Codons of the TM domain are doubly underlined, and exons boundaries [13] are indicated Sol-1 designates the corresponding sequence of the smaller product of about 260 bp (soluble IFNAR cDNA form 1, bold characters), interrupted by deletion (B) Translational frameshift and predicted C-terminus of soluble IFNAR form 1 (sol-1) compared to transmembranal IFNAR (TM)

primers revealed that the deletion is around the TM region Thus, with primers 2 and 3 (spanning nucleotides 1,342 to 1,515, Fig. 1), U266^{R} cDNA did not give any product, whereas the expected PCR product (173 bp) was clearly observed with U266^{S} cDNA (Fig 2, lanes 1 and 2). With primers 4 and 5 (spanning residues 1,522 to 1,749), the same unique PCR product was seen for both cell types (Fig. 2, lanes 5 and 6) indicating no heterogeneity in this region Likewise, PCR reactions with other primer pairs (sense 83–102 and antisense 553–572; sense 553–572 and antisense 1,270–1,289) confirmed that these cDNA segments containing the EC domain were similar in both cell types and yielded single PCR bands (not shown).

3 2 *Sequences of two IFNAR deleted transcripts*

In order to determine the nucleotide sequences of the large and small PCR DNA products, observed by amplification of U266^{S} cDNA with primers 2 and 5 (Fig. 2, lane 3), the two DNA segments were cloned (using prim-

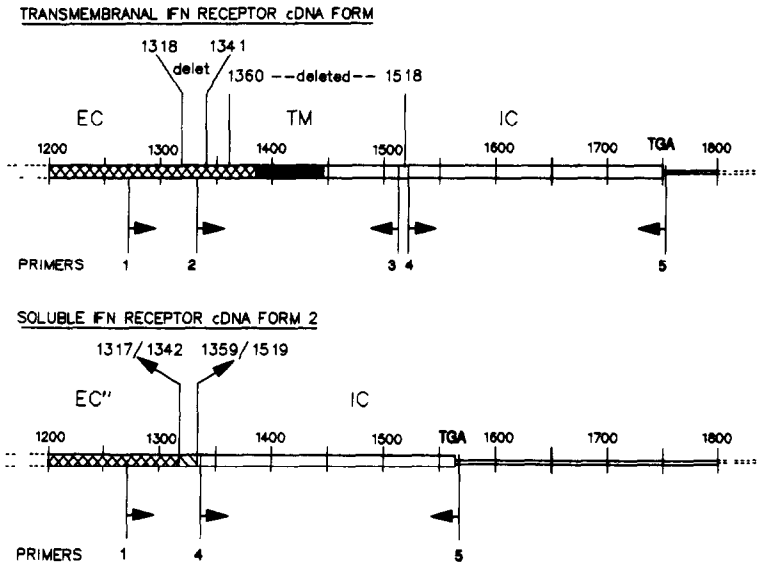

Fig 4 (upper) Scheme of part of transmembranal IFNAR cDNA (as in Fig 1), showing the double deletion in soluble IFNAR cDNA form 2, deduced from sequence in Fig 5 (lower) Scheme of same region of form 2 showing conservation of most of the IC domain (open box) The 6 residues conserved between the in-frame deletions are shown by single-hatched box

DELETION IN SOLUBLE FORM-2 OF IFNAR cDNA

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                        primer 1                1290                                    1320
TM                  CCA CTG ACT GTA TAT TGT GTG AAA GCC AGA GCA CAC ACC ATG GAT GAA AAG
Sol-2               CCA CTG ACT GTA TAT TGT GTG AAA GCC AGA GCA CAC ACC ATG GAT GAA
                     P   L   T   V   Y   C   V   K   A   R   A   H   T   M   D   E

                      primer 2a             1350                        exon IX | X  1380
TM          CTG AAT AAA AGC AGT GTT TTT AGT GAC GCT GTA TGT GAG AAA ACA AAA CCA GGA AAT ACC
Sol-2                               AGT GAC GCT GTA TGT GAG
                                     S   D   A   V   C   E

                                    1410                                            1440
TM          TCT AAA ATT TGG CTT ATA GTT GGA ATT TGT ATT GCA TTA TTT GCT CTC CCG TTT GTC ATT

                                    1470                                            1500
TM          TAT GCT GCG AAA GTC TTC TTG AGA TGC ATC AAT TAT GTC TTC TTT CCA TCA CTT AAA CCT

                      exon X  | XI            1530    primer 4                        1560
TM          TCT TCC AGT ATA GAT GAG TAT TTC TCT GAA CAG CCA TTG AAG AAT CTT CTG CTT TCA ACT
Sol-2                             TAT TTC TCT GAA CAG CCA TTG AAG AAT CTT CTG CTT TCA ACT
                                   Y   F   S   E   Q   P   L   K   N   L   L   L   S   T

                                    1590                                            1620
TM          TCT GAG GAA CAA ATC GAA AAA TGT TTC ATA ATT GAA AAT ATA AGC ACA ATT GCT ACA GTA
Sol-2       TCT GAG GAA CAA ATC GAA AAA TGT TTC ATA ATT GAA AAT ATA AGC ACA ATT GCT ACA GTA
             S   E   E   Q   I   E   K   C   F   I   I   E   N   I   S   T   I   A   T   V
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Fig 5 Partial nucleotide sequence of cloned PCR products made with primers 1 and 5a TM, sequence of large product (about 500 bp) identical to transmembranal IFNAR cDNA, with codons of the TM domain doubly underlined and exons boundaries Sol-2 (bold) designates the corresponding sequence of the smaller product of about 300 bp (soluble IFNAR cDNA form 2) with deduced amino acid sequence, interrupted by the two in-frame deletions

ers 2a and 5a with *Eco*RI and *Bam*HI tails, respectively) The sequence of resulting BlueScript plasmid DNAs with the larger insert of about 420 bp (Fig. 3) confirmed the known sequence for this region of the IFNAR cDNA, which contains exon IX/exon X boundaries, the TM domain in exon X and the exon X/exon XI boundary [7,13] In comparison, sequencing a number of plasmids with the smaller product of about 260 bp, showed a deletion from nucleotide 1,362 to 1,518 (157 bp) that removes the TM region (Fig 3) The identically deleted nucleotide sequence was found when the corresponding 260 bp PCR product from U266^R cDNA (Fig 2, lane 4) was analyzed The sequence reveals (Fig 3) that the deletion occurs toward the end of exon IX, which becomes spliced to the normal first residue of exon XI, thereby removing all of exon X containing the TM segment and the beginning of the IC domain This causes a frameshift after codon 427 (GLU) and predicts a truncated protein ending with 7 amino acids Asn-Ile-Ser-Leu-Asn-Ser-His (or S domain), not found in the transmembranal IFNAR (Fig 3, lower part) We designate this truncated protein, as soluble IFNAR form 1

Sequencing of PCR products produced with more upstream primers such as primer 1 containing nucleotides 1,270–1,290 (Fig 4), revealed yet another transcript of the IFNAR gene Thus, PCR reactions with the primer pair 1 and 5a, yielded two amplified bands of about 500 bp and 300 bp with U266^S cDNA, only the 300 bp product being obtained in the case of U266^R (not shown) The sequence obtained from 10 independent plasmids containing the smaller insert of about 300 bp, from the two cell types, showed two in-frame deletions of nucleotides 1,318 to 1,341 (in exon IX) and of nucleotides 1,360 to 1,518 (the end of exon IX and all of exon X), giving the soluble IFNAR form 2, shown in Fig 5 The 24 bp deletion from bases 1,318 to 1,341 explains (Fig 4) why this sequence was not detected, along with form 1, in the PCR products made with primer 2a (1,331–1,343) Conversely, we could demonstrate that the 300 bp PCR product of primers 1 and 5a, contained also the form 1 DNA because primer 2b corresponding to nucleotides 1,320–1,336 (deleted in form 2) hybridized to the 300 bp PCR product obtained with primers 1 and 5a pair, and allowed its secondary amplification yielding the form 1 sequence (not shown) As all the ten determined plasmid sequences of 300 bp products of primer 1 and 5a were form 2, we conclude that form 2 cDNA is more abundant than form 1 However, form 1 is detected if one uses a primer (such as 2a or 2b) which discriminates between the two forms

In comparison to the transmembranal IFNAR, the two in-frame deletions of the form 2 sequence (Fig 4) predict a protein in which the end of the EC domain is

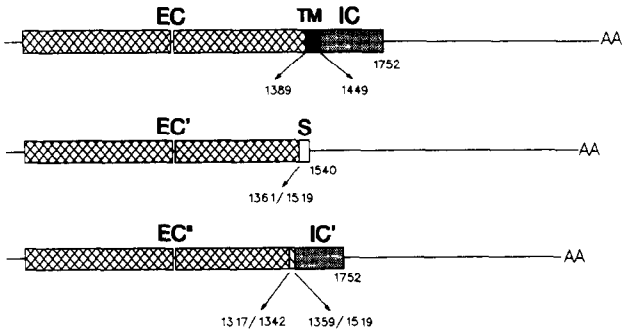

Fig 6 Schemes of three IFNAR cDNAs (upper) transmembranal form, (middle) soluble form 1, and (lower) soluble form 2 Boxes show EC, extracellular domain formed of two repeats [7], TM, transmembrane domain, IC, intracytoplasmatic domain, EC', EC", end-deleted EC domains of form 1 and form 2, S, novel tail of form 1, IC', remaining IC domain of form 2

deleted of 8 aa following Glu^{413}, conserves 6 aa (422–427 still in exon IX) and after a further deletion of 53 aa (including the TM domain) continues in the IC domain from Tyr^{481} The IC domain of form 2 conserves 77 aa of the 100 aa in the transmembranal form. It is worth noting that the 6 aa conserved in exon IX between the two deletions, contain a cysteine, thereby conserving the 8 cysteine residues found in the EC domain of the transmembranal IFNAR protein

4. Discussion

Receptors for many cytokines exist both bound to cell membranes and as soluble forms comprising the extracellular (ligand-binding) domain but truncated in a way which removes the transmembrane domain, thereby causing their release in body fluids (see [2] for review). For a number of receptor genes, the coexistence in the cells of transcripts encoding the transmembranal protein and of transcripts encoding corresponding soluble proteins has been shown by isolation of the respective cDNA forms. Transcripts for soluble receptors were identified for ligands such as IL-4 [14], IL-5 [15], IL-6 [6], IL-7 [16], G-CSF [17] and GM-CSF [5]. In most cases, these transcripts show a deletion of the mRNA region which encodes the transmembrane domain of the receptor, the deletion (or an insertion for the IL-4 receptor) causing a shift in the translated frame. Following the frameshift, the codons of the intracytoplasmatic domain are no more translated but the sequence encodes different aminoacids (whose number can be from 4 to 150) before reaching a termination codon (reviewed in [5]) The function, if any, of the novel amino acid tail in the soluble receptor forms has not been established, but expression of such frameshifted cDNAs does produce secretion of the soluble receptor from the cell [5]. Formation of soluble receptor transcripts, which seems compatible with an alternative processing or exon skipping, is a process different from shedding of truncated soluble receptor via proteolytic cleavage in which no new amino acids are added, such as for soluble Type I TNF [3] and p55 IL-2 receptors [18] The two processes may coexist, as for example in the case of the gp80 IL-6 receptor [4,6].

Soluble forms of the type I IFN receptor IFNAR of different sizes, have been detected in the blood and urine by specific antibodies and IFN-α binding [8]. By PCR analysis of cDNAs, we show the existence of transcripts from the IFNAR gene which encode two different soluble receptor proteins (form 1 and form 2) which both lack the TM domain (Fig. 6). Compared to the transmembranal cDNA sequence [7,13], form 1 has a single deletion from the end of exon IX to the normal splice acceptor residue of exon XI, producing a frameshift and a tail of 7 amino acids. This resembles the alternative processing found for other cell secreted cytokine receptors [5,6]. In contrast, the more abundant form 2 has the unique feature to conserve most of the C-terminal part of the IC domain (77 aa out of 100 aa, and 3 out of the 4 tyrosines), despite two in-frame deletions, one in exon IX and one taking away the end of exon IX and all exon X containing the TM domain The signalling function of the IC domain, which may involve tyrosine phosphorylation [19], could be conserved in this second soluble IFN receptor form. We have observed that upon expression in COS-7 cells of form 2 cDNA (but not of form 1), the protein product is detected inside the cell, in the soluble cytoplasm compartment (not shown). Whether form 2 could respond to endogenously produced IFN or to internalized IFN, or interact with other cellular signalling proteins remains to be investigated. Form 1, resembling other cell secreted soluble receptors [5] could function outside the cell. Both form 1 and form 2 conserve the 8 cysteine residues of the EC domain, 4 cysteines being thought to be involved in folding of each of the two repeats characterizing the ligand-binding domain in the EC part of IFNAR [7] No evidence for deletions in the EC domain was found. Therefore, the two natural, cell-synthesized, soluble IFNAR forms described here should retain ligand-binding activity, as do recombinant IFNAR EC domains [9] The potential function of soluble receptors as regulators of cytokine activities have been discussed [2], and the soluble IFNAR molecules could be interesting regulators for the antiviral, antiproliferative or immunoregulatory functions of Type I IFN-α,β.

The production of the transmembranal and soluble IFNAR transcripts, appears to be regulated since it differs in the two human myeloma cells studied The $U266^S$ cells contain abundant transmembranal IFNAR mRNA along with the form 1 and form 2 soluble IFNAR transcripts The $U266^R$ cells have lost the transmembranal IFNAR mRNA but clearly contain the form 1 and form 2 mRNAs. The $U266^R$ cells lack the membranal IFNAR protein and show no specific cell binding of exogenously added human IFN-α-A compared to 5,000 high affinity binding sites per cell for human IFN-α-A in $U266^S$ cells (Lundgren E. et al., unpublished data) Some molecular response of $U266^R$ cells to IFN can, nevertheless, still be detected (Abramovich et al , in preparation) The defect in $U266^R$ cells leading to absence of the IFNAR transcripts with all its 11 exons but allowing formation of alternatively processed transcripts without exon X, will be interesting to investigate as well as the function the soluble IFNAR form 1 and form 2 could play in these cells.

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